Institute of Paper Science and Technology
Atlanta, Georgia

IPST Technical Paper Series Number 692

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December 1997

Submitted to
Plant Molecular Biology Reporter

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Rapid and Reliable Differential Display from Minute Amounts of Tissue: Mass Cloning and Characterization of Differentially Expressed Genes from Loblolly Pine Embryos

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Abstract

Performing RNA differential display analysis on small tissue samples is difficult because much RNA, the initial template for the reaction, is lost during conventional isolation procedures. We have developed a rapid method that employs oligo-dT beads to capture mRNA from cell lysates. Subsequent reactions are primed directly from the beads; thus, RT and PCR reactions can be completed within a few hours of tissue harvest. This approach allows us to perform differential display on a single pine embryo. We describe strategies for distinguishing classes of comigrating bands excised from differential display gels and outline a PCR-based method for confirming differential expression of large numbers of cloned bands in cases where RNA quantities are limiting.

Introduction:

Somatic embryogenesis is one of the most promising approaches for the rapid propagation of high-quality forest trees. Although somatic embryos can now be produced in many species through plant tissue culture, the efficiency is still too low for commercial use (Attree *et al.*, 1993). Currently, no molecular markers are available to follow embryo development in trees and few are available in other plants (Sterk *et al.*, 1991). The goal of identifying genes expressed at different stages of somatic and zygotic embryo development in loblolly pine could be achieved by RNA differential display (Liang and Pardee, 1992). This method has the advantages of technical simplicity, lower bias against rare messages, and a requirement for lower quantities of mRNA starting materials compared to methods such as construction and screening of cDNA libraries. Nonetheless , the amount of RNA required in traditional differential display limits its application in cases where tissue is limited. A further frustration can arise due to the presence of large amounts of polysaccharides and other interfering substances that co-precipitate with RNA, especially in pine tissue. We have circumvented these problems by adopting a solid-phase approach for RNA differential display. Using this method, we were able to generate clear banding patterns using as little as a single embryo. The method is rapid; PCR products are ready for gel analysis within 6 hours of harvesting tissue.

A problem with cloning differential display bands is that the PCR products derived from the sequencing gel may be heterogeneous (Welsh *et al.*, 1992) and a cloned cDNA may not represent the differentially expressed gene of interest. Usually, northern blotting, using the cloned cDNA as probe, is needed to confirm the pattern of expression. When large numbers of bands are to be evaluated, this approach would be tedious and would require large amount of RNA, an impossible requirement for tissues such as early stage embryos. Dot-blots (Callard *et al.*, 1994; Corton and Gustafsson, 1997) or slot-blots (Liu and Raghothama, 1996) have been used to screen the cDNAs representing differentially expressed genes. These methods all require relatively large amounts of mRNA for making probes. We have modified the dot-blot strategies, reducing the requirement for RNA, by employing RT-PCR to amplify the messages. In this fashion, we were able to verify the expression of hundreds of genes in a single embryo.

Materials and Methods:

Plant Materials:

Zygotic Embryos: Loblolly pine (*Pinus taeda*) cones were collected weekly from Boise Cascade's breeding orchard near Lake Charles, Louisiana. Cones from tree BC-1 were shipped on ice, and seeds were collected for isolation of embryos. Seeds were cracked using a hemostat, pried open with the aid of a scalpel, and the integument and nucellus tissue removed from the ovule. The female gametophyte was slit, pried open, and the dominant embryo or mass or embryos removed. Embryos were quickly observed through a dissecting microscope and evaluated for stage (Pullman and Webb, 1994). Stage 9 embryos were also categorized by the week they were collected; 9.1 (Stage 9, week 1) 9.2 (Stage 9, week 2), etc. Staged zygotic embryos were then placed in a cryostorage vial partially emersed in liquid nitrogen. Twenty similar staged embryos were collected per vial. Frozen embryos were stored at -70°C until analyses were performed.

Somatic Embryo Development: Cultures of somatic embryos for loblolly pine were initiated as described by Becwar *et al.* (1990) or with modifications in media mineral composition. Somatic embryos were grown in cell suspension culture medium 16 and maturation medium 240 (Pullman and Webb, 1994). Resulting somatic embryos were selected, staged, and sorted into vials containing the same stage. Somatic embryos were stored at -70°C until analyses were performed.

Northern Blotting:

Total RNA was extracted from somatic embryos at suspension stage and stage 9, respectively, according to Chang *et al.* (1993). The RNA was denatured and separated in a agarose-formaldehyde gel according to Ausubel *et al.* (1987). Hybridization was carried out as for Southern blotting described in the protocol below.

Protocols:

Differential Display:

- Place 10-100 mg of staged embryos in a 1.5 ml tube; quickly add 50-100 µl Lysis Buffer (containing 100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% SDS and 5 mM DTT) and grind thoroughly with an electric drill. The drill 'bit' is a plastic pestle (VWR, Cat# KT95050-99) that has been autoclaved.

- Add another 50-100 µl Lysis Buffer, grind briefly, and use 100 µl Lysis Buffer to wash the grinder and vortex. If multiple samples are being processed, store them on ice until ready for the next step.

- Wash the grinding tip with sterile water and dry it with a paper towel for next sample.

- After all the samples are ground, spin at 4°C for 15 minutes in a bench top centrifuge at 14,000 rpm.

- Place 8 µl oligo(dT) coated Dynal beads (from mRNA DIRECT kit, Dynal, NY) in a 1.5 ml tube. The beads are washed twice with 100 µl Lysis Buffer and suspended in an equal volume of the Lysis Buffer used in tissue grinding. If more than one sample is handled, the beads for all the samples can be washed together and dispensed in several 1.5-ml tubes.

- Add the cleared embryo lysate (after centrifugation) to the beads and mix well.

- Incubate on ice for 5 minutes, place on a magnetic stand (Promega) for 5 minutes then carefully remove the liquid.

- Add 100 µl of Washing Buffer with LiDS (mRNA DIRECT kit, containing 100 mM Tris-HCl, pH 8.0, 0.15 mM LiCl, 1.0 mM EDTA, and 0.1% SDS) and transfer the mix to a 200 µl PCR tube.

- Wash the beads once with 100 µl Washing Buffer with LiDS and once with 50 µl Washing Buffer (mRNA DIRECT kit, containing 100 mM Tris-HCl, pH 8.0, 0.15 mM LiCl, and 1.0 mM EDTA).

- Wash the beads quickly with 20 µl 1x RT Buffer (25 mM Tris-HCl, pH 8.3, 37.6 mM KCl, 2.5 mM $MgCl_2$, and 5 mM DTT).

- Add 20 µl RT Mix containing 1X RT Buffer and 20 µM dNTP. Heat the tube at 65°C for 5 minutes, cool to 37°C, add 1 µl MMLV reverse transcriptase (Promega) and incubate at 37°C for 1 h. with occasional shaking.

- Add 20 µl of water to the RT reaction, mix and take 1.0 µl to 20 µl of PCR mix containing 1x Perkin-Elmer PCR buffer, 2.0 µM dNTP, 1.0 µM $T_{12}VN$, 0.2 µM arbitrary 10-mer, 1 unit *AmpliTaq* (Perkin-Elmer), 50 µCi α^{35}S-dATP (Amersham). Perform PCR using temperature settings of 94°C 30", 40°C 1', 72°C 2', 40 cycles, and 72°C 10' extension. (Perkin Elmer 9600 Thermal Cycler)

cDNA cloning of Differential Display Bands:

- Mark dried gel with radioactive ink prior to exposure to X-ray film.
- Align X-ray film to the dried gel plate. Mark the bands of interest by puncturing film with a pin so as to mark the gel underneath.
- Use a scalpel blade to score the gel around the band to be excised.
- Place 2 µl water in a PCR tube and 0.3-0.5 µl water onto the gel piece within the cutting lines, use the scalpel blade tip to scrape it up and transfer the gel to the PCR tube by touching the water drop in it.
- Perform PCR using 50 µl PCR mix that is the same as used for differential display with the following modifications: the concentration of the primers is increased to 1 µM, and dNTP concentration is increased to 200 µM; no ^{35}S-dATP is added. The cycling setting is the same as used for differential display.
- Run a portion of the PCR products on a gel to check the quantitiy and the size of PCR products, and discard any PCR that does not correspond to the size of the original differential display band.
- Purify the remaining PCR products using CHROMA SPIN-100 columns (Clontech) according to the manufacturer's instructions.
- PCR fragments were cloned into the pCR2.1 TA cloning vector (Invitrogen) and components of this kit were employed. The cDNA is in a great molar excess compared to the vector (over 100-fold) as suggested by Invitrogen protocols; however, multiple insertion has not been a problem. The ligation is done at 16°C overnight.
- Transform *E. coli* strain DH5α with the ligation, plate on LB plate with X-gal/IPTG.
- Perform PCR on five colonies, select PCR products of colonies with inserts of expected size. About 10 µl of 30 µl PCR reaction is then double-digested with Nla III, plus Mse I. Digestions were carried out at 37°C for 5 hour or overnight. Other enzymes with 4 base pair recognition sites have also be employed successfully.

- Select cDNA clones according to the colony PCR and the restriction enzyme digestion pattern.

Dot Southern:

Spotting Samples and Prehybridization:

- Amplify the cloned cDNA fragments by PCR and adjust to equal concentration.
- Dispense 1.65 µl of 3.0 M NaOH into PCR tubes, add 15 µl of PCR DNA, and incubate at 65°C for 30'.
- Cool to room temperature and add 20.5 µl 20xSSPE.
- Spot 0.5 µl on membrane (Hybond-N+, Amersham).
- Crosslink the DNA to membrane (120,000 mJ/cm^2 in a CL-1000 UV-linker, Upland, CA).
- Prehybridize the membrane in Hybridization Buffer (0.5 M Na-phosphate, pH 7.2, 5% SDS, and 10 mM EDTA) at 65°C for 30'.
- Remove the prehybridization solution, add 3 ml new Hybridization Buffer, add denatured probes to the hybridization bottle, and hybridize in the hybridization oven at 65°C overnight.
- Check the radioactivity on the membranes, wrap them with plastic wrap, and expose them to Bio-Max film (Kodak).

Probe Preparation and Hybridization:

- Set up PCR reactions for making probes: H_2O, 22.5 ml; buffer (Perkin-Elmer #1), 5 ml; dATP+dGTP+dTTP (5 mM each), 5 ml; T12MN (0.04 mM each), 5 ml; AP primer (2 mM), 5 ml; template (RT-beads), 2 ml; *Taq*, 0.5 ml; ^{32}P-dCTP, 5 ml. When cDNAs to be evaluated originate from several primer pairs these primer pairs, are added to the PCR reaction.
- Perform PCR using conditions: 94°C 4' and 40 cycles of 94°C 30", 40°C 1', 72°C, 2 minutes.
- Purify the probes using NICK column (Pharmacia, cat# 17-0855-02), and denature the probes by heating at 95°C for 4' and cooling on ice immediately.
- Remove the prehybridization solution, add 3 ml new Hybridization Buffer, add the probes to the hybridization bottle, and hybridize in the hybridization oven at 65°C overnight.

- Wash the membranes with 0.1x Hybridization Buffer at room temperature three times.

- Check the radioactivity of the membranes, wrap them with plastic wrap, and expose them to Bio-Max film.

N.B.: We have recently synthesized probes using the SMART PCR cDNA synthesis system (Clontech, CA). In this protocol, mRNA is isolated from tissue using the bead method and eluted from beads. This method uses end-linked primers to amplify 'full-length' copies of mRNA. A complete representation of mRNA should result which will be suitable for evaluating cDNAs generated by a variety of primer pairs. In our hands results are very clean as shown in Fig.4.

Results and Discussion:

Differential Display:

Although total RNA extracted from abundant tissues is suitable for differential display, this approach is time consuming and usually requires large amounts of starting material. Magnetic bead separation of RNA has been employed in mRNA isolation, and the isolated mRNA has been used in differential display (McKendree *et al.*, 1995; Rosok *et al.*, 1996). However, to investigate the gene expression in early stage embryos, we need a more sensitive and reliable method. We have developed a procedure that is less time consuming and more sensitive. From harvest of starting material to the end of PCR, our new procedure takes less than 6 hours. The protocol may be accelerated still further by the use of high-speed, air-cycling thermal cyclers (Idaho Technologies Inc., ID).

The most important feature of the new method we describe is its sensitivity. Figure 1 shows the differential display generated by using 1, 2, 5 and 10 zygotic embryos of loblolly pine. As little as 1 embryo (~10 mg fresh weight) gave clear banding patterns almost identical to those generated when more embryos were used (reactions were continued to saturation point in all cases). Because only $1/40^{th}$ of the RT reaction was used in the PCR phase of differential display, the RT from a single embryo could be used in differential display for up to 40 primer sets. When the differential display products of different stage embryos were run side by side on a sequencing gel, the changing expressions of some genes across the developmental stages are apparent (Fig. 2). When different batches of embryos at the stage were used in the differential display, then band patterns were similar (data not shown). We do not perform DNase digestion of nucleic acid before RT, however, DNA contamination is not a problem with these protocols, and prior treatment with DNase had no effect on our results (data not shown).

Compared to the previously reported solid-phase differential display (Rosok *et al.*, 1996), the new method offers several additional advantages. The use of oligo(dT) beads instead of streptavidin beads coupled with biotin-labeled T12VN shortened mRNA isolation time by eliminating the steps involved in preparing the streptavidin-biotin conjugates. The poly(T) on the oligo$(dT)_{25}$ beads is longer than that of the T12 as in T12VN, and this should increase mRNA isolation efficiency because of enhanced binding. Most importantly, because d(T) instead of TnVN was used for the priming in the RT reaction, any anchored primers can be used in the subsequent differential display PCR reactions.

Few beads were used in our method. The Dynal oligo(dT) beads can bind to 2 μg mRNA/ml. When a small amount of tissue is used, most of the oligo(dT) is wasted and may interfere with the subsequent PCR reaction. We reduced the bead quantity from the manufacturer's recommendation of 50 μl/sample to 8 μl/sample, an amount that is barely visible in the tube during isolation. If a larger amount of tissue is used, the amount of oligo(dT) beads can be increased.

Cloning:

The Invitrogen TA Cloning Kit (Invitrogen, CA) is convenient for cloning PCR products; however, at least in our hands, the system gives variable results. When PCR product was used directly in the ligation, as recommended by the manufacturer, and transformed into *E.coli*, many white or light blue colonies with no detectable insert were observed. We now size-purify the PCR product using CHROMA SPIN columns (Clontech, CA) and use the purified DNA for ligation. This greatly reduces the number of light blue transformants, and most of the white clones have inserts.

The DNA amplified from the differential display bands may be heterogeneous in sequence. This heterogeneity was examined by restriction enzyme digestion. After the DNA from a differential display band was cloned in the pCR2.1 vector and the insert was amplified by PCR, two 4-cutters, *Mse I* and *Nla III* were used to digest the insert DNA from different clones. Different band patterns on the gel of the digestion revealed the difference in sequence (Fig 3).

Southern:

False positives are common problems in RNA differential display (Liang *et al.*, 1992; Sun *et al.*, 1994), and northern blotting is usually required to confirm differential expression of cloned DNA fragments. This is impossible for us to carry out because our goal is to clone hundreds of the differentially expressed gene fragments, and the amount of plant material we are working with is very small. To circumvent this problem, we chose high-density, dot-blotting reverse Southern analysis. The cloned DNAs were dot-blotted on membranes at a density of 2.5 dot/cm^2 and hybridized to cDNA probes made from the embryos at different stages. The probes are made by PCR in the presence of ^{32}P-dCTP using the first strand cDNA templates, which were generated from single or a few embryos using the SMART PCR cDNA Synthesis Kit (Clontech, CA). Figure 4 shows the results of the Southern blots using probes made from suspension stage and stage 9 somatic embryos, respectively. There were 96 cloned cDNA fragments blotted on each of the membranes, and 70 of them were cloned from differential display bands present either at suspension stage or at stage 9. The stage appearance of 49 of them corresponded to those on the differential display gel.

We also performed northern analyses (Fig. 5) on a few selected cDNA fragments to check if the result of the Dot-Blot-Southern agrees with that of northern (for suspension culture and stage 9 embryos, it is possible to isolate relatively large amounts of RNA, compared to intermediate stages). Our results showed that both methods gave similar results for both the pattern and level of expression.

Conclusion:

This package of procedures offers a fast, sensitive and reliable approach to the isolation and study of the expression of many genes that are differentially expressed during development. The 'Southern' analysis would permit characterized clones to be arrayed and used to screen for expression in a fashion similar to 'chip' technologies being developed (Schena, 1996).

Acknowledgements:

We would like to acknowledge the support of the Georgia Consortium for Technological Competitiveness in Pulp and Paper and the Member Companies of the Institute of Paper Science and Technology.

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Figure Legends:

Figure 1: RNA differential display of a single embryo of loblolly pine. Poly(A) RNA was isolated from 1, 2, 5, or 10 stage 9.7 zygotic embryos (lanes 1, 2, 3, 4, respectively), and RT-PCR performed according to the procedures outlined in the Protocol. The embryos were from tree BC-1. T12MC (where M represents a degenerate base, A, C, or G) and 5'-GGTACTCCAC-3' primers were used in the PCR.

Figure 2: Differential display band patterns of somatic embryos of loblolly pine. The stages of the embryos are on the top, and S represents suspension stage, which covers both stage 1 and stage 2. T12MC (where M represents a degenerate base, A, C, or G) and 5'-GGTACTCCAC-3' primers were used in the PCR for the left panel, and T12MG and 5'-GGTACTCCAC-3' for the right panel.

Figure 3: Graphic presentation of the cloning protocol for isolated differential display bands.

Figure 4: Dot-blot reverse Southern analysis. Sections a and b are schematics of the procedures used. Section C shows the result of two blots using probes made from embryos at suspension stage and stage 9, respectively. LPS001 to LPS096 cDNAs were cloned from differential display of somatic embryos of all stages. The insert sequences were amplified by PCR and blotted on the membrane. The order of the dots is from upper-left (LPS001), going downward to the lower-right (LPS096).

Figure 5: Northern blotting analysis of selected cDNAs. Lanes S and 9 contain 5 μg of total RNA isolated from suspension stage and stage 9 somatic embryos. The probes were made from late stage expression cDNA LPS017, LPS034, early stage expression cDNA LPS056, and both stage expression (at a lower level) cDNA LPS059, respectively. See Figure 4 for the expression level detected by reverse Southern.

Fig.1

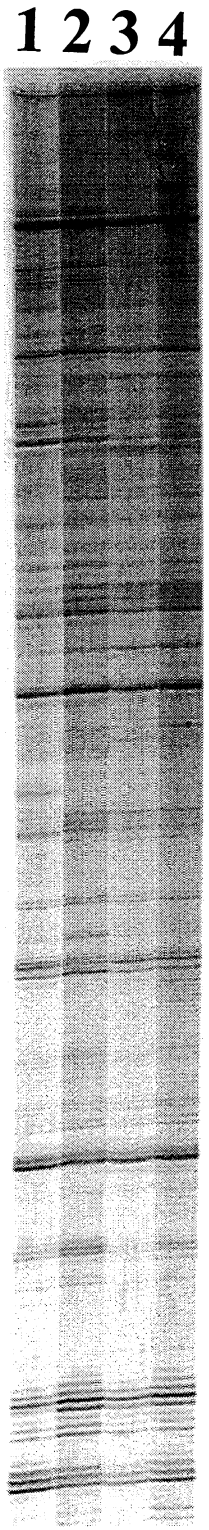


Fig.2

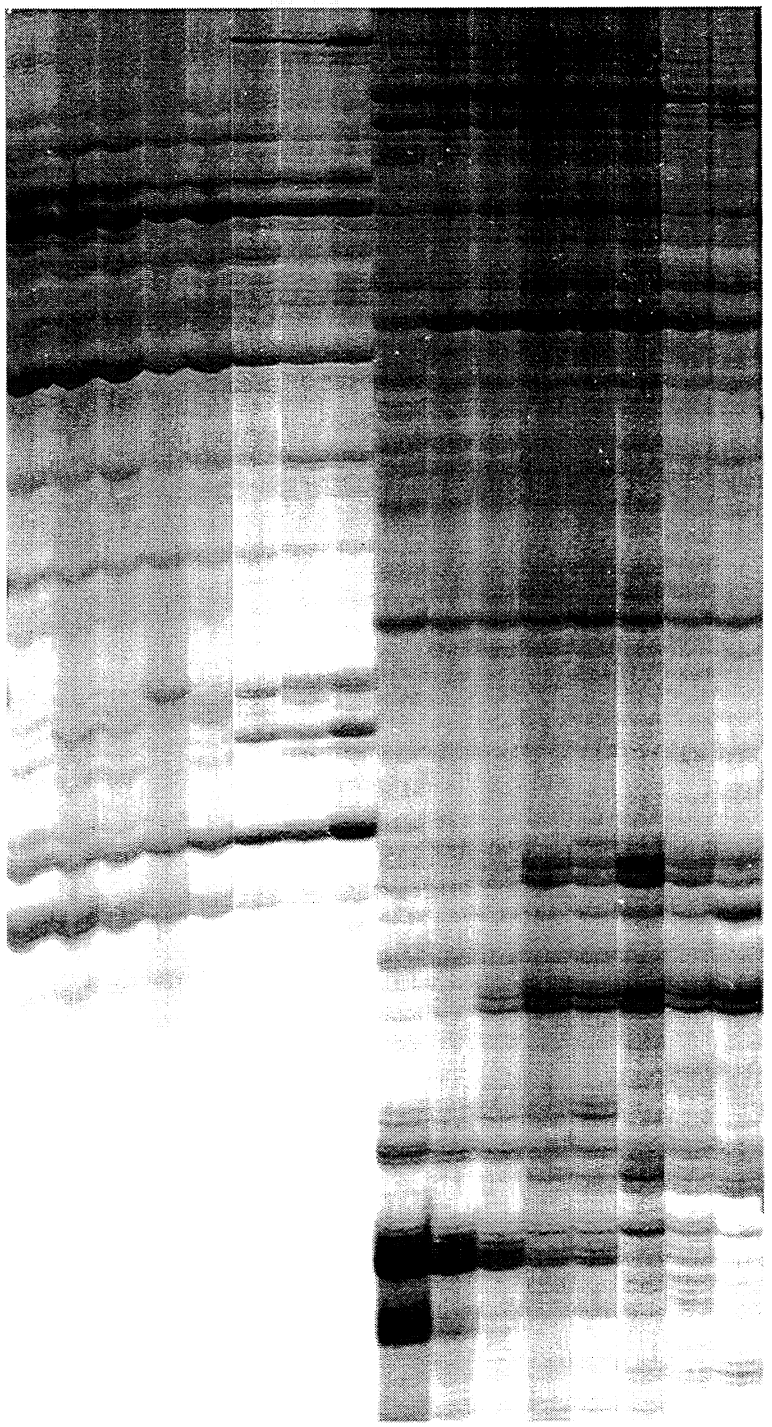
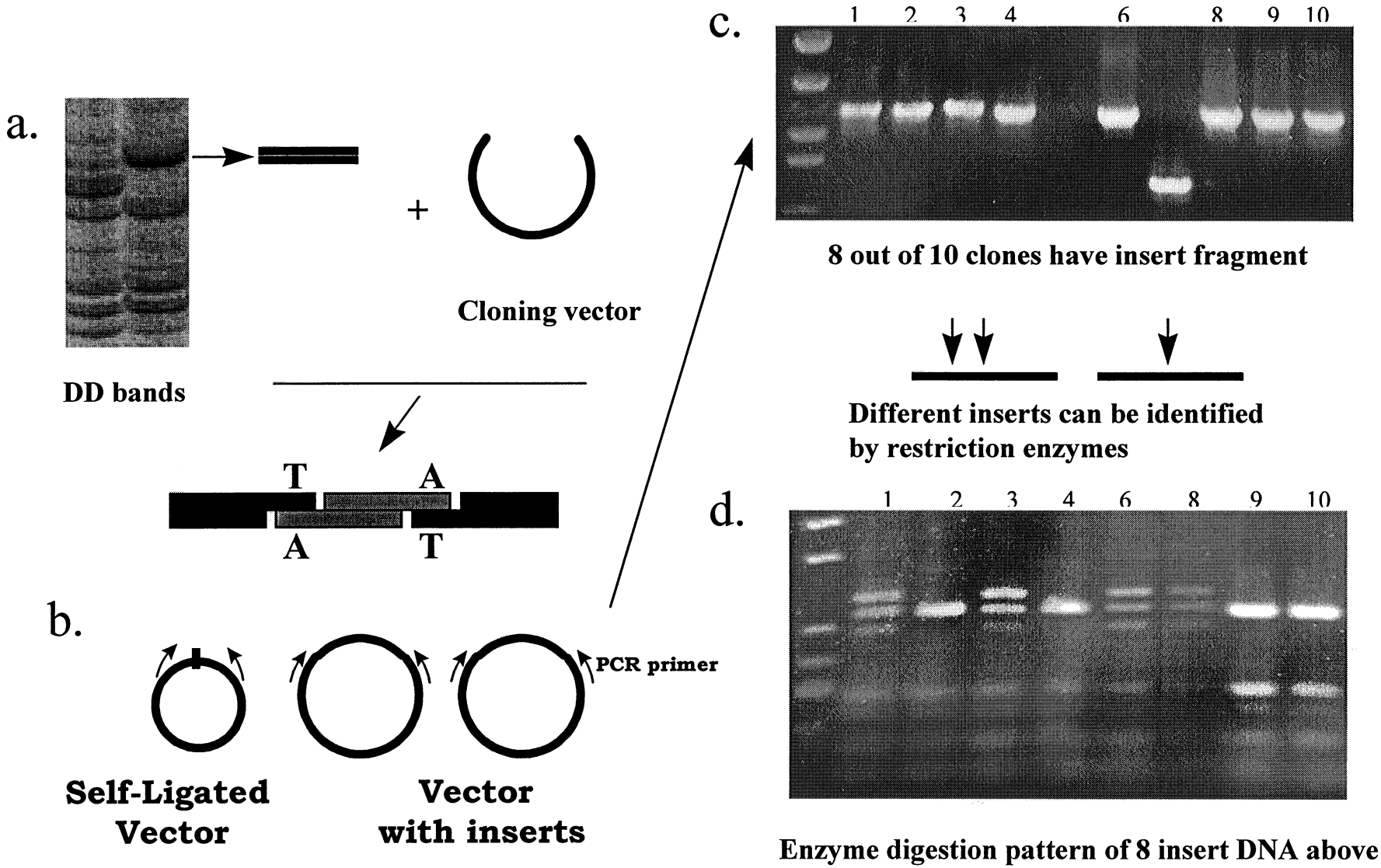


Fig.3


a.
+
Cloning vector
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b.
PCR primer
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Vector with inserts
c.
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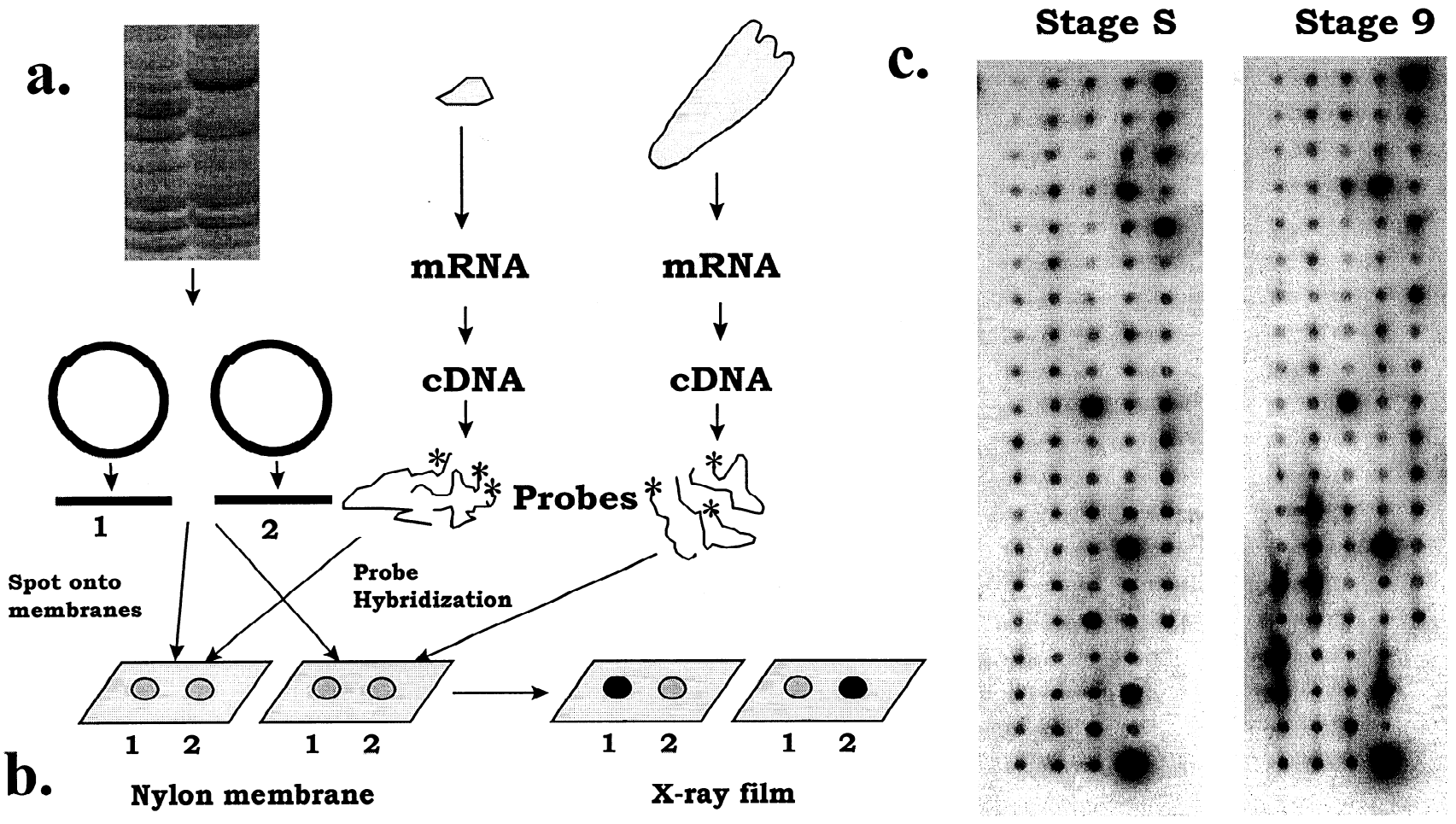
8 out of 10 clones have insert fragment
Different inserts can be identified by restriction enzymes
d.
1 2 3 4 6 8 9 10
Enzyme digestion pattern of 8 insert DNA above

Fig. 4


a.
mRNA
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1
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Spot onto membranes
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X-ray film
c.
Stage S
Stage 9

Fig. 5